EVOSEP

Making clinical proteomics
100 times more robust
and 10 times faster

Simple, robust and ultra-sensitive proteomics analysis

Evosep One is a standardized omics solution designed to provide reproducible results, reduce system overhead, simplify sample preparation and increase robustness.

With the addition of Whisper™ flow technology, very small sample amounts, down to a single cell, can be robustly and confidently identified with the high sensitivity from true nano-flow.

"The Evosep One has been pivotal to scale real single cell analysis robustly to thousands of measurements, making it an ideal and standardized platform for future biomarker discovery work."

Professor Matthias Mann
Group Leader at the Max Planck Institute of Biochemistry, Proteomics and Signal Tranduction

To learn more about Evosep, the Evosep One instrument and Whisper flow technology, follow the link below.

www.evosep.com

Proteomics
Proteomics and Systems Biology
www.proteomics-journal.com

Quantitative Proteomic Analysis of Seminal Plasma, Sperm Membrane Proteins, and Seminal Extracellular Vesicles Suggests Vesicular Mechanisms Aid in the Removal and Addition of Proteins to the Ram Sperm Membrane

Tamara Leahy, Jessica P. Rickard, Taylor Pini, Bart M. Gadella, and Simon P. de Graaf*

Quantitative proteomic studies are contributing greatly to the understanding of the spermatozoon through the provision of detailed information on the proteins spermatozoa acquire and shed in the acquisition of fertility. Extracellular vesicles (EVs) are thought to aid in the delivery of proteins to spermatozoa in the male reproductive tract. The aim of this study is to isolate, identify and quantify EV proteins isolated from ram seminal plasma. Ram sperm plasma membrane proteins are also isolated using nitrogen cavitation and identified to better understand the interplay of proteins between the sperm membrane and extracellular environment. The categorization of proteins enriched in the EV population according to their function revealed three main groupings: vesicle biogenesis, metabolism, and membrane adhesion and remodeling. The latter group contains many reproduction-specific proteins that show demonstrable links to sperm fertility. Many of these membrane-bound proteins show testicular expression and are shed from the sperm surface during epididymal maturation (e.g., testis expressed 101; TEX101 and lymphocyte Antigen 6 Family Member K; LY6K). Their association with seminal EVs suggests that EVs may not only deliver protein cargo to spermatozoa but also assist in the removal of proteins from the sperm membrane.

1. Introduction

During transit through the male reproductive tract, at ejaculation, and upon entry to the female tract, spermatozoa communicate with, and are modified by, factors in their extracellular environment. The complex and varied protein complement of reproductive fluids orchestrates the simultaneous uptake, release, and modification of proteins at the sperm surface.[1] The reproductive luminal proteome is tightly controlled by the secretion and absorption of proteins from epithelial cells lining the reproductive tract. Luminal proteins may contain an N-terminal signal peptide that indicates that they are secreted in a so-called classical manner via the translocation apparatus of the endoplasmic reticulum.[2] However, many reproductive luminal proteins lack this feature and utilize alternate, or non-classical, secretion pathways. Very hydrophobic proteins, often containing a transmembrane domain or glycosylphosphatidylinisotol (GPI) anchor, are present in the luminal fluid of the male reproductive tract.[3] Such proteins are not soluble in a hydrophilic environment but have been shown to be transported in the luminal fluid to the sperm surface where they can even exhibit membrane-like properties.[4,5] Small membrane bound vesicles were determined to be the source of this unique intercellular communication system.[6–8] The extracellular release of protein-rich vesicles (broadly termed extracellular vesicles (EVs)) represented a strategic system in which non-soluble proteins could be transferred to the sperm surface. Vesicular transport even allows for the possibility of entire protein complexes, such as the ubiquitin–proteasome system[9] or glycolytic machinery[10] to be secreted into the lumen and transferred to spermatozoa in a functional state. As spermatozoa are transcriptionally inert cells that are dependent on proteins in their environment for sperm maturation and survival, extracellular vesicles appear to be an elegant and targeted means of protein delivery.

The most abundant proteins in epididymal membrane vesicles (epididysomes) have now been described in the man,[8]

Dr. T. Leahy, Dr. J. P. Rickard, Dr. S. P. de Graaf
School of Life and Environmental Sciences
Faculty of Science
University of Sydney
Sydney, New South Wales 2006, Australia
E-mail: tamaraleahy@gmail.com
Dr. T. Pini
Colorado Center for Reproductive Medicine
Lone Tree Colorado 80124, USA
Dr. B. M. Gadella
Department of Farm Animal Health and Department of Biochemistry and Cell Biology
Faculty of Veterinary Medicine
Utrecht University
Yalelaan 2, CM Utrecht 3584, The Netherlands

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/pmic.201900289

DOI: 10.1002/pmic.201900289

bull,[11–13] mouse,[14] and ram.[15] Seminal extracellular vesicles in human semen are also one of the most functionally characterized extracellular vesicle populations due to the pioneering work of the Ronquist laboratory from the 1970s to the present day.[16,17] This vesicle population is often referred to as prostasomes as the predominant contributor of vesicles in human seminal plasma (SP) is the prostate. The protein complement of human prostasomes have been extensively described and functionally defined.[18–21] Prostasomes have been reported to both inhibit or stimulate sperm motility, capacitation, and the acrosome reaction and provide immunosuppressive and antibacterial protection for spermatozoa [for review see [17,22,23]). The influence of seminal extracellular vesicles in other species is not so well defined. In the ram, the effect of seminal plasma on sperm function and fertility has been extensively investigated and reviewed[24–27] but the contribution of seminal vesicles has received minimal attention.[28] The ability to characterize and quantify the protein composition of ram seminal extracellular vesicles will help us to decode their functional role. In this study, we seek to understand if any proteins transferred to the ram sperm surface at ejaculation are likely to be of EV origin and what the broad function of ram seminal vesicles may be. Comprehensive quantitative analysis of proteins in ram seminal plasma and purified seminal vesicles is used to help decode vesicle biogenesis, origin, and function. Qualitative analysis of proteins of the ram sperm membrane are included to assess possible uptake of vesicular proteins by ram spermatozoa.[29]

Significance Statement

Extracellular vesicles (EVs) are secreted phospholipid and protein constructs assist in intracellular communication. In the male reproductive tract, EVs contribute to sperm maturation by transporting proteins between epithelial cells and spermatozoa. However, fertilization competence also requires the shedding of numerous proteins from the sperm membrane as it transits the male tract, though the mechanism/s underlying the release of these proteins is not known.

In the current study, SWATH analysis was used to assess which proteins were not just present in the seminal plasma derived EV population but were significantly enriched in the EV population, compared to whole seminal plasma. This technique identified seminal proteins that may be transferred from the extracellular fluid to the sperm membrane at the point of ejaculation via vesicular means (e.g., EDIL3). The seminal EV population was also highly enriched in testicular proteins that are shed from the sperm surface during epididymal maturation (e.g., TEX101, LY6K) to prepare the spermatozoa for fertilization. The identification of these proteins in the EV population of the lower male reproductive tract suggests EVs may also assist in the removal of proteins from the sperm membrane and provides proteins of interest for the targeted assessment of this phenomenon.

2. Experimental Section

2.1. Chemicals and Media

Unless otherwise stated, products were sourced from Sigma-Aldrich (Castle Hill, NSW, Australia) and were of the highest reagent grade available.

2.2. Semen Collection

Mature merino rams ($n = 3$) and ewes ($n = 2$) used for semen collection were housed at the University of Sydney, Camperdown campus. Animals were maintained on a chaff based diet, supplemented with lupins. All procedures were approved by the University of Sydney animal ethics committee (approval 2013/5854). Ejaculates were collected by artificial vagina from Merino rams ($n = 3$) in the presence of a teaser ewe. Ejaculates were immediately assessed for wave motion (data not shown), and only accepted if the wave motion scored 4 or higher out of 5. For this study two ejaculates were collected from the three Merino rams twice weekly for 4 weeks in the breeding season. Of the 48 ejaculates collected one was discarded for having a wave motion score lower than 4.

2.3. Preparation of Seminal Plasma and Isolation of Extracellular Vesicles

The 47 ejaculates, collected from 3 rams, were pooled and centrifuged at 16 000 × *g* for 20 min at room temperature within 15 min of collection. All further procedures were performed at 4 °C by pre-cooling the rotors, centrifuges, and diluents used. The supernatant was collected and centrifuged again (16 000 × *g*, 20 min) and the resulting seminal plasma supernatant stored at –80 °C.

Extracellular vesicles were isolated from seminal plasma by centrifugation at 100 000 g for 90 min in a fixed rotor (Hitachi CP100NX Ultracentrifuge). The pellet was washed with 12 mL of Hepes Buffered Saline (HBS; 5 mM Hepes, 150 mM NaCl, pH 7.4) which was supplemented with complete ultra mini EDTA-free protease inhibitor tablet (Roche Diagnostic) and centrifuged again at 100 000 g for 90 min. The resulting pellet was resuspended in 1 mL of HBS.

Optiprep was used to isolate the extracellular vesicle population as previously described.[30] Optiprep was diluted with a 0.25 M sucrose, 10 mM Tris (pH 7.5) solution to generate 40, 20, 10, and 5% iodixanol solutions. A discontinuous iodixanol gradient was prepared by placing 3 mL of the 40% solution in a tube and layering 3 mL of the 20%, 10%, and 5% solutions on top, followed by 1 mL of the washed extracellular vesicle pellet. The gradient was centrifuged for 16 h at 103 000 g in a spin-out rotor (Hitachi CP100NX Ultracentrifuge). A large band at the 10/20% interface (denoted Band 1, B1) and a smaller band at the 20/40% interface (denoted Band 2, B2) were collected (**Figure 1**). The bands were washed with 12 mL of HBS and centrifuged again at 100 000 g for 2 h. The pellets were resuspended in 150 µL of HBS and stored at −80 °C until further use for mass spectrometry studies.

2.4. Characterization of Extracellular Vesicles Size and Concentration by Nanoparticle Tracking Analysis

The Brownian motion of the vesicles was analyzed using a NanoSight NS300 (Malvern Panalytical) to determine vesicle size

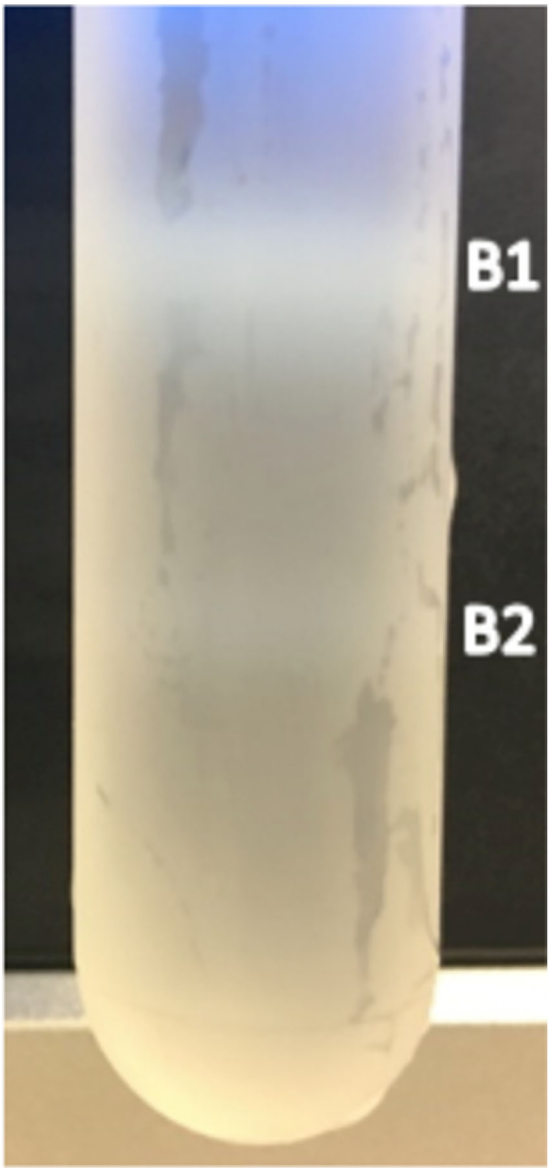

Figure 1. Density gradient sub-fractionation of ram seminal EVs into an abundant lighter density band (Band 1; B1) and a less abundant higher density population (Band 2; B2).

and concentration. The samples were measured for 60 s at 25 °C. Samples were diluted with Milli-Q water to obtain an optimal concentration of 50–100 particles per view. The shutter was adjusted manually and gain was equal for all the measurements. The data was analyzed using the Nanoparticle Tracking Analysis 3.4 software (NanoSight). Three measurements were performed for each sample and the particle concentration of the blank (Milli-Q only) was deducted from the sample concentration.

2.5. Isolation of Sperm Plasma Membrane Vesicles

Sperm ejaculates were washed on a BoviPure gradient (Nidacon International) as per manufacturer's instructions to remove seminal plasma, dead sperm and debris. A standard nitrogen cavitation and differential centrifugation protocol was followed for isolation of the plasma membrane vesicles.[31,32] Six cavitations were performed using two ejaculates from three rams. Briefly, 1×10^9 washed spermatozoa in TBSS (Tris buffered sucrose solution; 5 mM Tris, 250 mM sucrose, pH 7.4) were subjected to nitrogen cavitation at 650 psi for 15 min at 22 °C in a cell disruption device (Parr Instruments, Moline, IL, USA). The cavitate was slowly extruded and the cell disruption device was washed with 1 mL of TBSS supplemented with protease inhibitors (TBSS+). The cavitate and wash were centrifuged at $1000 \times g$ for 10 min (22 °C) and the supernatant was recovered and further clarified at $11\,000 \times g$ for 10 min (4 °C). Plasma membrane vesicles were pelleted from the resultant supernatant by ultracentrifugation (P80AT fixed rotor, Hitachi centrifuge) for 1 h at $232\,000 \times g$ (4 °C). The pellet containing the plasma membrane vesicles (PMV) was re-suspended in HBS (HEPES buffered saline; 5 mM HEPES, 150 mM NaCl, pH 7.4) and sonicated for 15 min in a ultrasonic bath (Bransonic, Branson, CT USA) to disperse the sticky membrane material from the bottom of the tube. The PMV isolates were further sub-fractionated by sucrose density centrifugation[33,34] using a three step discontinuous gradient containing 1.0, 1.30, and 1.57 M sucrose in 5 mM Tris pH 7.4 for 2 h at $103\,040 \times g$ (4 °C). The PMVs sedimented between 1.0 and 1.30 M sucrose. The bands in this region were collected, pooled and diluted in HBS then centrifuged at $232\,000 \times g$ for 1 h. The pellets were resuspended in HBS and frozen in aliquots at −80 °C.

2.6. Protein Preparation for Mass Spectrometry by Filter Aided Sample Preparation

Sperm PMVs, seminal plasma (SP) and extracellular vesicles (EVs) from B1 and B2 were processed with a filter aided sample preparation kit (Expedeon Inc, San Diego, CA) as previously described.[35] Briefly, protein concentration was determined by Qubit protein assay (2.0 fluorometer; Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions and 50 µg of protein was SDS-solubilized (Tris 62.6 mM, 5% SDS, complete ultra mini EDTA-free protease inhibitor tablet, pH 6.8). The solubilized samples were reduced with 10 mM TCEP (1 h, 37 °C) and mixed with the UA buffer (8 M urea, 0.1 M Tris-HCl, pH 8.5) in a 10 kDa cut-off Microcon filter (Millipore, Watford, UK). The device was centrifuged at $14\,000 \times g$ at 20 °C for 15 min. Successive washes and alkylation steps were performed. The washed concentrate was then subjected to proteolytic digestion in the absence of urea, using modified sequencing grade trypsin (Promega, Madison, WI, USA). Tryptic peptides were eluted after digestion on the filter. The peptides were acidified (1% formic acid) and quantified (Qubit protein assay) and 1 µg aliquots vacuum dried prior to mass spectrometry.

2.7. Quantitative Proteomic Comparison of Proteins in Whole SP and Extracellular Vesicles Populations

2.7.1. Generation of an Ion Spectral Library using LC-MS/MS

Whole SP was used as the global standard for the generation of an ion spectral library. 1 µg/6 µL of seminal plasma protein was

separated by nano-LC using an Eksigent 415 UHPLC system (Sciex) coupled to an in-house built fritless nano 75 µm (i.d.) × 20 cm column packed with ReproSil Pur 120 C18 stationary phase (1.9 µm particle size, Dr. Maisch GmbH, Germany). LC mobile phase buffers were comprised of A: 0.1% (v/v) formic acid and B: 80% (v/v) acetonitrile, 0.1% (v/v) formic acid. Peptides were eluted using a linear gradient of 5% to 40% B over 90 min and then 95% B wash over 1 min at a flow rate of 300 nL min^{-1}. Mass spectra were acquired in the mass-to-charge ratio (m/z) range of 350–1, 500 using a 6600 TripleTOF mass spectrometer (Sciex, Foster City, USA). Up to 50 of the most abundant ions, with charge states between +2 to +5 were sequentially isolated and fragmented, and a product ion scan collected over 100–1800 m/z. Ions selected for MS/MS were dynamically excluded for 20 s.

2.7.2. LC-MS/MS of Whole SP and Extracellular Vesicles Populations Employing SWATH Acquisition

One microgram of each sample was separated by nano-LC using the same 90 min gradient conditions described above. MS/MS runs were conducted using a 6600 TripleTOF mass spectrometer (Ab Sciex, Foster City, USA) in data independent acquisition mode, using SWATH acquisition. A total of 34 windows at a width of 54 Da covered the mass range 300–1500 Da. An overlap of 1 Da between each SWATH window was used. An accumulation time of 96 ms was used for each fragment ion scan and for the survey scans acquired at the beginning of each cycle, resulting in a total cycle time of 3.3 s. The rolling collision energy for each window was 2+. The mass spectrometer was operated in high sensitivity mode. Two technical reps were performed.

2.7.3. Peptide Identification by Comparison to Ion Spectral Library

An ion spectral library was generated by searching LC results (.wiff format) of the global standard in Proteinpilot (v5.0, Sciex), using the Paragon search algorithm. Search parameters included iodoacetamide as an alkylating agent, trypsin as a protease, thorough ID search effort and a detected protein threshold of 0.05. A unique ion spectral library was produced by searching the global standard against an NCBI database for all mammals (downloaded August 2016), with enrichment for *Ovis aries*.

Identification of proteins was performed in the SWATH microapp within Peak View (v1.2.0.4; Sciex). The ion spectral library of interest was imported (.group format), excluding shared peptides. Retention time calibration was performed by selecting house-keeping proteins spaced equally along the 90 min gradient, with high intensity (>2500) of the fragment ion, no modifications and a charge state of +1. Samples were compared to the relevant ion spectral library using the following processing parameters; five peptides per protein, six transitions per peptide, 90% peptide confidence, exclusion of shared peptides, XIC extraction width 5 min and XIC width 0.1 Da.

Protein values were considered significantly different if they met two conditions 1) a t-test of their mean values returned a significantly different ($P < 0.05$) result and 2) the fold-change of their mean values was 2 or greater.

2.8. Qualitative 2D LC-MS/MS Analysis of Proteins in Sperm Plasma Membrane Vesicles

Ten micrograms of PMVs were pooled, dried down, and resuspended in 90% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. Peptides were first fractionated using an Agilent 1200 HPLC system coupled to an in house built fritless TSK-Amide 80 HILIC 4.5 mm (i.d.) × 17 cm column (5 µm particle size). LC mobile phase buffers were comprised of A: 0.1% (v/v) trifluoroacetic acid (TFA) and B: 90% (v/v) acetonitrile, 0.1% (v/v) TFA. A total of 12 fractions were eluted into a V bottom 96 well polypropylene plate (Griener, Sigma Aldrich, Castle Hill, Australia) using 100% B for 26.5 min, followed by a linear gradient of 60–30% B over 11 min, and then 100% B wash over 2 min at a flow rate of 6 µL min^{-1}. The resultant fractions were dried down and resuspended in 3% (v/v) acetonitrile, 0.1% (v/v) formic acid ready for LC-MS/MS using the same 90 min gradient conditions described in previously in "Generation of an ion spectral library using LC-MS/MS." All mass spectrometry proteomic data has been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017537.

2.9. Statistical Analysis

Proteins with a false discovery rate below 1% were exported into Markerview (v1.3.1, Sciex) for statistical analysis. A t-test (α of 0.05) was used to compare protein differences between SP, EVB1, and EVB2 samples and a high fold change (>2 or <2) was applied as a further cut off to ensure statistical significance.

3. Results

3.1. Physical Characterization of Extracellular Vesicle Populations in Ram Seminal Plasma

Two distinct extracellular vesicle sub-fractions, B1, and B2, were present after Optiprep sucrose density discontinuous gradient centrifugation of the seminal extracellular vesicle pellet (Figure 1). B1 vesicles separated as a profuse, homogenous, band of lighter density at the 10/20% interface. B2 vesicles separated at the 20/40% interface, indicating they were denser than the B1 vesicle population. B2 was more diffuse and contained considerably less vesicular material then B1.

The size distribution of EVs pelleted from whole seminal plasma and the B1 and B2 subfractions was analyzed by nanoparticle tracking analysis (NTA; Nanosight) and the concentration of particles in whole seminal plasma was assessed (**Figure 2**). Ram seminal plasma had a particle concentration of $\approx 995 \times 10^9$ particles per mL. Seminal plasma had a mean vesicle population size of 136.9 ± 61.5 nm (Figure 2A) and showed a normal distribution in which 10% of the sample was smaller at the 77.7 nm mark (D10), 50% was smaller at the 129.8 nm mark (D50; midpoint)

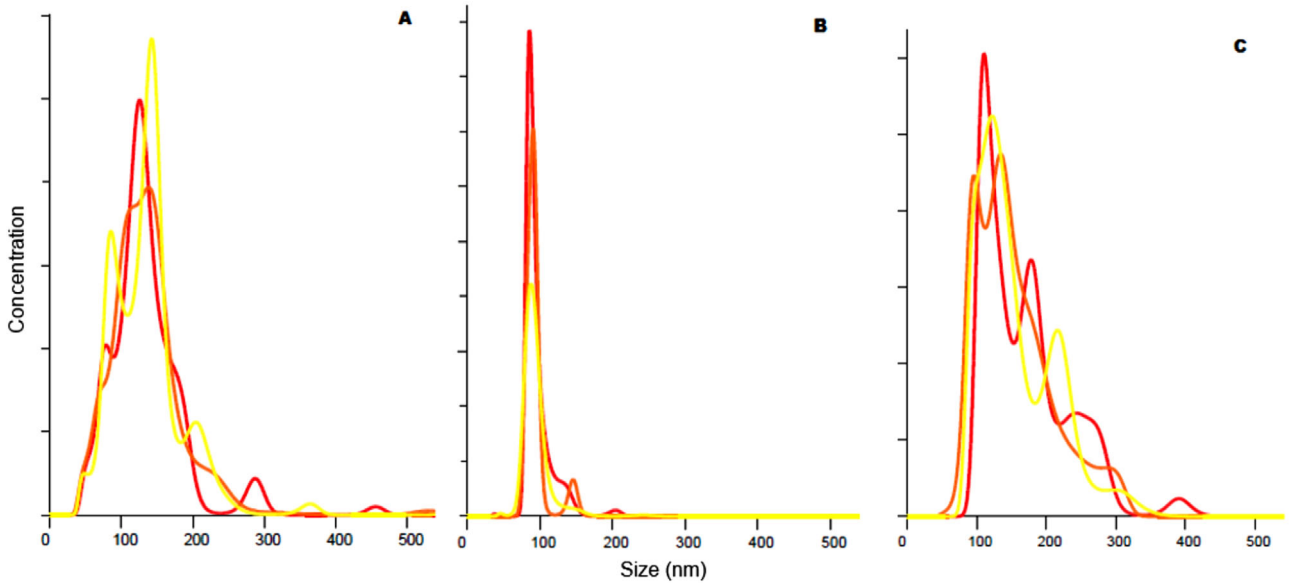

Figure 2. Representative schematic of the size distribution of nanoparticles in A) whole ram seminal plasma and B) purified Band 1 and C) Band 2 ram seminal extracellular vesicles. Nanoparticle tracking analysis was performed using NanoSight NS300 and three replicates are displayed (represented by different colors).

and 90% was smaller at the 192.5 nm mark (D90). B1 vesicles had a mean vesicle population size of 95.1 ± 19.0 nm (Figure 2B) and a particle size distribution in which 10% of the sample was smaller at the 79.2 nm mark (D10), 50% was smaller at the 89.5 mark (midpoint) and 90% was smaller at the 114.7 mark (D90). B1 vesicles had one peak size with minimal variation. The particle size distribution of B2 vesicles was skewed to the right, due to a population with larger particles, or possible aggregates of smaller particles (Figure 2C). The mean vesicle population size was 160.4 ± 58.7 nm and showed a particle size distribution in which 10% of the sample was smaller at the 97.8 nm mark (D10), 50% was smaller at the 144.1 mark (midpoint), and 90% was smaller at the 243.7 mark (D90).

3.2. Quantitative Proteomic Comparison of the Two Ram Seminal Extracellular Vesicle Populations (B1 versus B2)

The proteins in the two extracellular vesicle bands (B1 and B2 on sucrose gradient; Figure 1) were quantitatively compared to identify proteins that were enriched in a particular population. Quantitative analysis was performed on 520 proteins identified in these protein preparations and the data can be found in File 1, Supporting Information. While the two extracellular vesicle preparations shared many common proteins at similar quantitative levels there were some striking differences. Those showing greater abundance in B2 included seminal plasma proteins and sperm proteins involved in folding and proteolysis.

Highly abundant serum seminal plasma proteins which were more highly represented in B2 compared to B1 include caltrin (PYY2; elevenfold), C-type natriuretic peptide (NPPCL; fourfold), proteins of the cysteine-rich secretory protein family (CRISP1, CRISP3; tenfold and threefold), binder of sperm proteins (BSPH1; 15-fold), and spermadhesins (SPADH1L, SPADH2L; 25 and 19-fold). These proteins are not likely to be of vesicular origin and are found in far higher abundance in whole seminal plasma compared to the extracellular preparations (Files 2 and 3, Supporting Information). However, the prevalence of secretory seminal plasma proteins in B2 may indicate the extracellular vesicles in this preparation originated from the accessory sex organs.

B2 also showed large, and mostly universal, increases in the abundance of proteins involved in protein folding (chaperones) and protein degradation (proteolytic proteins) compared to B1. All identified heat shock proteins (HSP90AA1, HSP90B1, HSPA13, HSPA1L, HSPA4, HSPA4L, HSPA5) were found at higher levels in B2 compared to B1 and these differences were up to 25-fold in magnitude. All proteins identified in the chaperonin-containing TCP1 complex (CCT; CCT2, CCT4, CCT6A, CCT6B, and CCT8) were also found at higher levels in B2 (four to seven-fold difference). B2 also had higher levels (two to 14-fold) of proteins which function as positive (STUB1, UBA1, CUL3, BAG6) or negative (UCHL3, UCHL7, USP7) regulators of protein degradation through the ubiquitination or deubiquitination of target proteins. The 26S proteasome complex showed a more intriguing localization pattern across the two extracellular vesicle samples. This large proteomic complex degrades ubiquitin tagged proteins and consists of a 20S core particle and two 19S regulatory subunits. Proteins associated with the 20S core particle were two to fourfold higher in B1 (PSMA2, PSMA3, PSMA8, PSMB1, PSMB5, PSMB6, PSMB7) while proteins associated with the 19S regulatory units were four to 14-fold higher in B2 (PSMC1, PSMC3, PSMC5, PSMD2, PSMD3, PSMD4, PSMD6, PSMD7, PSMD12, PSMD13). The reason for this is unclear but the most likely explanation is that B2 contains more cellular debris as most of these proteins have a sperm origin. Despite this, their presence in ram seminal plasma is routinely reported and the abundance of such proteins in ram seminal plasma has been

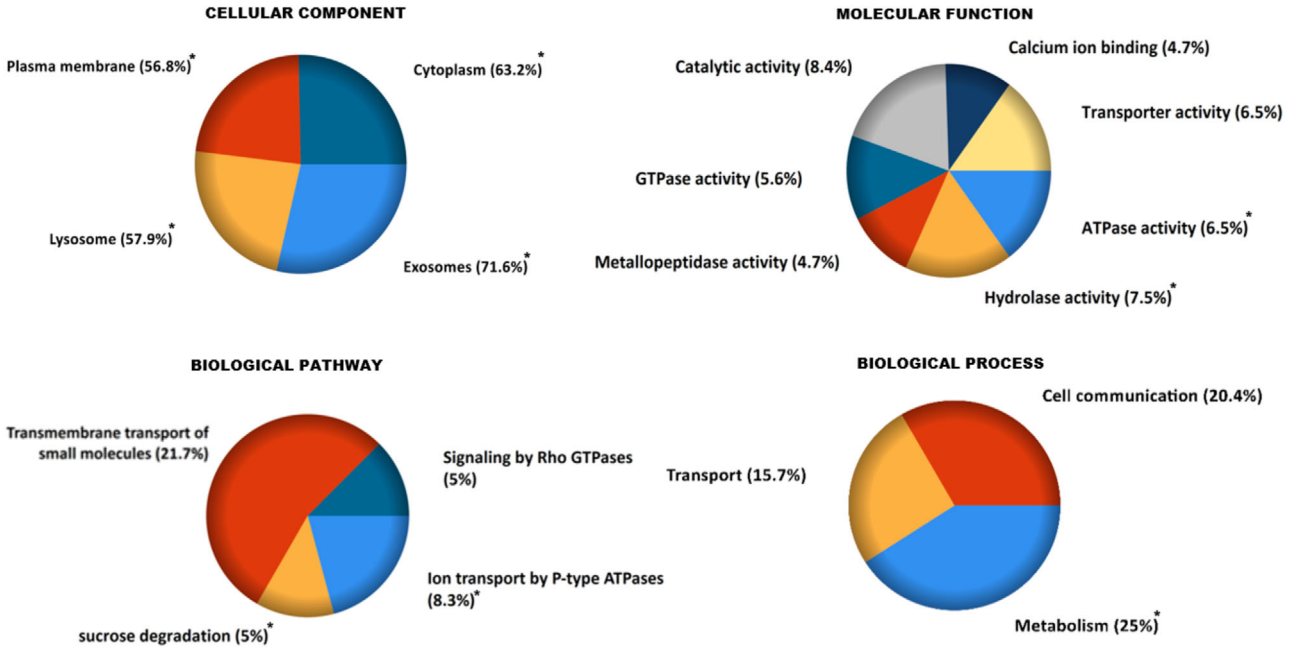

Figure 3. Gene ontology analysis of the proteins enriched in Band 1 ram seminal extracellular vesicles (EVs) was performed by FunRich 3.0 (Functional Enrichment analysis tool, http://www.funrich.org/). Enriched terms were ranked by *p*-value (hypergeometric test) using FunRich. A $p < 0.05$ was considered significant and is denoted by an asterisks (*).

correlated to in vitro sperm function.[36,37] Other sperm-associated proteins which are upregulated in B2 compared to B1 include zona pellucida binding protein (ZPBP; 47-fold), epididymal-specific lipocalin-12 (LCN12; 40-fold), the protease PRSS50 (30-fold), acrosin (ACR; ninefold), and acrosin-binding protein (16-fold).

Taken together these results show that B2 contains a more heterogeneous population of extracellular vesicle proteins than B1. B2 has a higher abundance of proteins which are major secretory seminal plasma proteins and are not likely to be of vesicular origin (e.g., BSP proteins, caltrin). Proteins that are likely to have originated from spermatozoa (e.g., 26s proteasome, acrosin) are also more abundant in B2 compared to B1. This suggests this vesicular prep may be more highly contaminated with proteins from its immediate environment. For this reason, for the comparative analysis with seminal plasma we focused on the proteomic conditions of B1 which appeared to be a cleaner extracellular vesicle preparation. However, quantitative comparison of the proteins in B2 with whole seminal plasma can be found in File 3, Supporting Information.

3.3. Quantitative Proteomic Comparison of ram Seminal Extracellular Vesicle Population B1 and Seminal Plasma (B1 versus SP)

Quantitative analysis was performed on 520 proteins in the extracellular vesicle and whole seminal plasma protein preparations and the raw data can be found in File 2, Supporting Information and **Table 1**. Proteins with increased abundance in seminal plasma were predominantly soluble, secreted, serum proteins. Cysteine-rich secretory proteins (CRISP1 23.3 and CRISP2 5.2-fold) and binder of sperm proteins (BSP5 20.0-fold) were more abundant in seminal plasma as were general serum proteins such as osteopontin (SPP1; 7.1), ceruloplasmin (CP; 12.3), and c-type natriuretic peptide (NPPCL; 250-fold). The predominance of secreted proteins in the seminal plasma population is also shown through the high number (71%) of proteins predicted to contain a signal peptide (SignalP; File 2, Supporting Information) and undergo classical secretion. Seminal plasma proteins were also enriched in protease and peptidase inhibitors. Notable examples include TIMP2 (111.1), A2M (90.9), CST6 (35.7), SPINT4 (2.9), SPINK9 (23.3), and SEPINA11 (5.8).

Proteins of increased abundance in the extracellular vesicle population included ADAM proteins and proteins involved in metabolism and extracellular vesicle biogenesis. Gene ontology and network analysis programs, including FunRICH, DAVID and vesiclepedia, were used to extensively characterize these ram seminal EV proteins and the results are detailed below.

3.4. Functional Enrichment Analysis and Domain Prediction of the Extracellular Vesicle Population (B1)

Cellular component analysis of ram seminal EVs in FunRich revealed significant ($P < 0.001$) over-representation of GO terms associated with exosomes, lysosome, cytoplasm, and plasma membrane (**Figure 3**). Computational prediction software (SignalP, TMHMM, SecretomeP, and PredGPI) also revealed a strong bias toward plasma membrane associated proteins (File 2, Supporting Information). Seminal EV proteins were predicted to undergo classical (prediction of a signal peptide) or non-classical secretion

Table 1. Proteins identified by LC-MS/MS, and quantified by SWATH, which were present in significantly higher quantities in extracellular vesicles (Band 1) compared to whole seminal plasma.[a)]

GI Number[b)]	Gene symbol[c)]	Name[d)]	Fold change[e)]	Sperm protein[f)]	Unique EV[g)]	Domains[h)]	Function[i)]
803246627	SYNGR4	Synaptogyrin-4 isoform X3	278	*	*	NCS, TM	
803256578	EZR	Ezrin isoform X2	136			NCS	Cytoskeletal anchoring at membrane
966003978	GNB2	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 isoform X1	131				Signal transduction
965931500	SYPL1	Synaptophysin-like protein 1 isoform X1	120	*		NCS, TM	Transport
803005032	MME	Neprilysin isoform X1	110	*		NCS, TM	Proteolysis
426258725	S100A14	Protein S100-A14	95			NCS	Protein binding
667481346	DPP4	Dipeptidylpeptidase 4	71	*		TM	Proteolysis
803291520	PROM2	Prominin-2 isoform X3	70	*		SP, TM	Protein phosphorylation
820867038	CDC42	Cell division cycle 42	69			NCS, GPI	Protein binding
965981203	BASP1	Brain acid soluble protein 1 isoform X1	68	*		NCS	Protein binding
803324131	ENPP3	Ectonucleotide pyrophos-phatase/phosphodiesterase family member 3	60			NCS	Metabolic process
426257139	RP2	Protein XRP2	60	*		NCS	Post-Golgi vesicle-mediated transport
965923257	CD9	CD9 antigen isoform X1	55			TM	Cell adhesion
965999604	CNDP2	Cytosolic non-specific dipeptidase isoform X1	50				Metabolic process
426249102	ENTPD3	Ectonucleoside triphosphate diphosphohydrolase 3	45			TM	
965941287	SLC9B1	Sodium/hydrogen exchanger 9B1 isoform X1	41	*	*	NCS, TM	Hydrogen ion transport
965920023	ADAM7	Disintegrin and metalloproteinase domain-containing protein 7 isoform X1	38			SP, TM	Proteolysis
261244978	PRDX2	Peroxiredoxin-2	36			NCS	Response to ROS
426243081	ELSPBP1	Epididymal sperm-binding protein 1	36	*		SP	Single fertilization
548452748	RAB5A	Ras-related protein Rab-5A	34	*		NCS	Synaptic vesicle recycling
803309500	GGT1	Gamma-glutamyltranspeptidase 1 isoform X2	34			TM	Glutamate metabolic process
426251447	NAALAD2	N-acetylated-alpha-linked acidic dipeptidase 2 isoform X1	33			TM	Proteolysis
802986340	GSTM3	Glutathione S-transferase Mu 3	31	*			Glutathione metabolic process
803014695	VAMP3	Vesicle-associated membrane protein 3	31	*		NCS, TM	SNARE complex assembly
803333382	ATP11C	Phospholipid-transporting ATPase IG isoform X14	30	*		NCS, TM	Phospholipid translocation
965985376	MFGE8	Lactadherin isoform X1	29	*		SP	Cell adhesion
803268039	BASP1	Brain acid soluble protein 1 isoform X2	29	*		NCS	Protein binding
240849225	AK1	Adenylate kinase isoenzyme 1	28	*		NCS	ATP metabolic process
803329230	PRSS42	Serine protease 42	28	*	*	SP, TM, GPI	Proteolysis
965829325	EFR3A	Protein EFR3 homolog A isoform X3	27	*			Localization to plasma membrane
803030711	SLC2A3	Solute carrier family 2, facilitated glucose transporter member 3 isoform X1	27	*		TM	Glucose transmembrane transport
803008531	TXNDC8	Thioredoxin domain-containing protein 8 isoform X5	27	*		NCS	Response to oxidative stress

(*Continued*)

Table 1. Continued

GI Number[b)]	Gene symbol[c)]	Name[d)]	Fold change[e)]	Sperm protein[f)]	Unique EV[g)]	Domains[h)]	Function[i)]
426238345	ARHGDIA	Tho GDP-dissociation inhibitor 1	27				GTPase activity
965945817	ACTC1	Actin, alpha cardiac muscle 1	26	*		NCS	ATPase activity
8809716	ACTB	Beta-actin, partial	26	*		NCS	Protein binding
803238129	*ADAM5L*	Disintegrin and metalloproteinase domain-containing protein 5-like	26	*	*	SP, TM	Proteolysis
426255175	*ABCA3L*	ATP-binding cassette sub-family A member 3-like isoform X1	25		*	NCS, TM	Fatty acid biosynthetic process
803222772	BSG	Basigin	25	*		SP, TM	Protein targeting to plasma membrane
426216262	SLC16A1	Monocarboxylate transporter 1	24	*		TM	Pyruvate metabolic process
965892847	ATP1A4	Sodium/potassium-transporting ATPase subunit alpha-4 isoform X2	24	*		NCS, TM	Ion transmembrane transport
965856700	ATP2B1	Plasma membrane calcium-transporting ATPase 1 isoform X3	23			TM	ATPase activity (GO:0005388)
803238742	GSTM1	Glutathione S-transferase Mu 1 isoform X3	23	*			Metabolic process
6166169	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	22	*		NCS	Canonical glycolysis
966002210	ABCA3	ATP-binding cassette sub-family A member 3 isoform X5	20	*		TM	Lipid transport
803249757	TPI1	Triosephosphate isomerase isoform X2	20	*			Gluconeogenesis
965908116	GLIPR1L1	GLIPR1-like protein 1	19	*	*	SP, GPI	
965943699	ANXA2	Annexin A2 isoform X2	19	*		NCS	Vesicle fusion
965967245	SLC6A9	Sodium- and chloride-dependent glycine transporter 1 isoform X3	19	*		NCS, TM	Amino acid transmembrane transport
803190469	LDHA	L-lactate dehydrogenase A chain isoform X1	19	*			Lactate metabolic process
803205273	GML	Glycosyl-phosphatidylinositol-anchored molecule-like protein isoform X1	19	*	*	SP, GPI	Negative regulation of cell proliferation
965925740	RHOA	Transforming protein RhoA isoform X1	19	*		NCS	Rho protein signal transduction
965912033	LY6K	Lymphocyte antigen 6K	19	*	*	SP, GPI	C-terminal protein lipidation
803263808	STXBP2	Syntaxin-binding protein 2 isoform X3	17	*			Vesicle docking involved in exocytosis
803204214	PLS3	Plastin-3 isoform X2	17				Actin filament bundle assembly
803256409	*PTCHD3L*	Patched domain-containing protein 3-like isoform X2	16	*	*	TM	Spermatid development
556752005	*ATP1B3L*	Sodium/potassium-transporting ATPase subunit beta-3-like isoform X1	16	*		NCS, TM	Cellular potassium ion homeostasis
803104786	RAB2A	Ras-related protein Rab-2A isoform X1	16	*			Protein transport
965912836	*ADAM20L*	Disintegrin and metalloproteinase domain-containing protein 20-like isoform X2	15		*	NCS, TM	
803210769	CMTM2	CKLF-like MARVEL transmembrane domain-containing protein 2	15	*	*	TM	Cytokine activity
965912829	*ATP2B4L*	Plasma membrane calcium-transporting ATPase 4-like isoform X2	15	*		TM	Transport
803280481	PRSS21	Testisin isoform X2	14		*	SP, GPI	

(*Continued*)

Table 1. Continued

GI Number[b)]	Gene symbol[c)]	Name[d)]	Fold change[e)]	Sperm protein[f)]	Unique EV[a)]	Domains[h)]	Function[i)]
965913208	*ADAM20L*	Disintegrin and metalloproteinase domain-containing protein 20-like	14	*	*	SP, TM	
803088378	PKM	Pyruvate kinase PKM isoform X6	14				Glycolytic process
803147285	LYPD4	Ly6/PLAUR domain-containing protein 4 isoform X2	13		*	NCS, GPI	C-terminal protein lipidation
78499349	RAB11A	Ras-related protein RAB11A	13	*		GPI	Establishment of vesicle localization
548476715	ANXA1	Annexin A1	12			NCS	Phospholipid binding
803239431	LCP1	Pastin-2	11				Extracellular matrix disassembly
965939677	EDIL3	EGF-like repeat and discoidin I-like domain-containing protein 3 isoform X2	11	*		NCS	Cell adhesion
426228047	AKR1B1	Aldose reductase	10				Carbohydrate metabolic process
803307323	IQGAP2	Ras GTPase-activating-like protein IQGAP2 isoform X3	9	*			Signal transduction
965820031	HK1	Hexokinase-1 isoform X5	9	*			Glycolytic process
803319553	TEX101	Testis-expressed sequence 101 protein	9	*	*	SP, GPI	C-terminal protein lipidation
426231623	UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	9			NCS	Protein deubiquitination
803015187	PRSS55	Serine protease 55	8	*		SP, GPI	Proteolysis
426222409	*ADAM20L*	Disintegrin and metalloproteinase domain-containing protein 20-like	8			TM	
965982917	STX2	Syntaxin-2 isoform X1	8	*		NCS	Synaptic vesicle fusion
803296352	VAT1	Synaptic vesicle membrane protein VAT-1 homolog isoform X2	8	*			
803307457	MROH2B	Maestro heat-like repeat-containing protein family member 2B isoform X2	8	*	*	NCS	Protein kinase A signaling
803279065	HTATIP2	Oxidoreductase HTATIP2	8			SP	Oxidation-reduction process
803322611	*ADAM1AL*	Disintegrin and metalloproteinase domain-containing protein 1a-like	8	*	*	TM, GPI	
426243615	DPEP3	Dipeptidase 3	7	*	*	SP, GPI	Proteolysis
426247306	PEBP1	Phosphatidylethanolamine-binding protein 1	7	*		NCS	Endopeptidase activity)
965878698	*CD46L*	Membrane cofactor protein-like	7	*		SP, GPI	Complement activation
530049	YWHAE	14-3-3 protein	7	*			Protein binding
965970296	*CEACAM21L*	Carcinoembryonic antigen-related cell adhesion molecule 21-like	6	*	*	SP, TM, GPI	
240849297	GDI2	Rab GDP dissociation inhibitor beta	6				Signal transduction
803201494	PPEF1	Serine/threonine-protein phosphatase with EF-hands 1 isoform X2	6	*	*		Protein dephosphorylation
803151934	LOC105602015	Uncharacterized protein LOC105602015 isoform X1	6		*	SP, GPI	
426246299	*HEXBL*	Beta-hexosaminidase subunit beta-like isoform X1	6			SP, TM	Lysosome organization
803238668	SORT1	Sortilin isoform X3	6	*		SP, TM	Endosome to lysosome transport
803337422	ACE	Angiotensin-converting enzyme isoform X3	6	*		SP, TM	Proteolysis
803240538	LAP3	Cytosol aminopeptidase	5				Proteolysis
807045877	LYPLA1	Acyl-protein thioesterase 1	5			NCS	Fatty acid metabolic process
803041597	TSPAN8	Tetraspanin-8 isoform X2	5	*		NCS, TM	Cell surface receptor signaling pathway

(*Continued*)

Table 1. Continued

GI Number[b)]	Gene symbol[c)]	Name[d)]	Fold change[e)]	Sperm protein[f)]	Unique EV[a)]	Domains[h)]	Function[i)]
803238131	ADAM2	Disintegrin and metalloproteinase domain-containing protein 2 isoform X5	4	*	*	TM	Fusion of sperm to egg plasma membrane
966002012	STUB1	E3 ubiquitin-protein ligase CHIP isoform X1	4	*		NCS	Ubiquitin-dependent catabolic process
965940741	ANXA5	Annexin A5 isoform X1	4			NCS	Signal transduction
426223797	GALM	Aldose 1-epimerase isoform X1	4				Glucose metabolic process
57164179	PGD	6-phosphogluconate dehydrogenase, decarboxylating	4				Pentose-phosphate shunt
426215552	ATP6V0D1	V-type proton ATPase subunit D	4				
803237559	ATP6V1B1	V-type proton ATPase subunit B, kidney isoform	4			NCS	Regulation of pH
803048315	LTA4H	Leukotriene A-4 hydrolase isoform X1	4			NCS	Proteolysis
256818752	IZUMO1	Izumo sperm-egg fusion protein 1 precursor	4	*	*	SP, TM, GPI	Cell adhesion
57164307	IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic	4			NCS	Glutathione metabolic process
426223462	MDH1	Malate dehydrogenase, cytoplasmic	3				Carbohydrate metabolic process
548520454	ALDOA	Fructose-bisphosphate aldolase A isoform X1	3	*			Glycolytic process
803317915	PCMT1	Protein-L-isoaspartate(D-aspartate) O-methyltransferase isoform X11	3	*		NCS	Protein methylation
803282033	ATP6V1A	V-type proton ATPase catalytic subunit A isoform X2	3	*			Transferrin transport
803131482	*PGAM1L*	Phosphoglycerate mutase 1-like	3				Glycolytic process
803322613	*ADAM1AL*	Disintegrin and metalloproteinase domain-containing protein 1a-like isoform X2	3	*	*	TM	
965909087	*WFDC6L*	WAP four-disulfide core domain protein 6A-like isoform X2	3		*	TM	
803108999	ESD	S-formylglutathione hydrolase	3			NCS	Glutathione biosynthetic process
803195647	HAGH	Hydroxyacylglutathione hydrolase, mitochondrial isoform X2	3	*		NCS	Pyruvate metabolic process
803146753	CAPNS1	Calpain small subunit 1 isoform X3	3			NCS	Extracellular matrix disassembly
803294734	DEFB127	Beta-defensin 127 isoform X2	3	*	*	SP, TM	Innate immune response
426234742	NT5E	5'-nucleotidase isoform X1	3			SP, GPI	NAD metabolic process

[a)] Protein identifications are based on at least 2 peptides. Only proteins showing significant quantitative differences ($P < 0.05$) that are threefold or greater are shown here for brevity purposes. Additional proteins, and more detailed information, is shown in File 2, Supporting Information; [b)] GI number, gene name and symbol are from the NCBI database. Genes names in italics with a "l" are not official gene symbols and indicate the gene is similar to, or"–like," the gene denoted; [c)] fold change is rounded to the nearest whole number; mean values are shown in File 2, Supporting Information; [d)] an asterisks (*) denotes protein was identified in the ram sperm membrane proteome shown in File 4, Supporting Information [g)] an asterisks (*) denotes protein was not identified in the Vesiclepedia database; [e)] Protein domain indicates predictions of a signal peptide (SP), transmembrane regions (TM) or non-classical secretion (NCS) from the SMART and THMM databases.

in 22% and 39% of cases, respectively. Transmembrane (35%) or lipid anchored proteins (GPI proteins; 15%) were also highly represented in the proteome. In total, 76% of seminal EV proteins were predicted to contain one of these extracellular localization signatures.

Molecular function analysis of ram seminal EVs in FunRich revealed significant ($P < 0.05$) over-representation of GO terms associated with catalytic activity and transmembrane transport (Figure 3). Enrichment in terms associated with active transport was seen through GO terms mapping to ATPase's, GTPase's, and other hydrolytic enzymes. ATPase's are membrane bound transporters that energetically transport substances across the plasma membrane. Seminal EVs contain ATPase's that transport lipids (ATP11C), sodium/potassium (ATP1A4, ATP1B3L), calcium (ATP2B4L), and protons (ATP6V0D1, ATP6V1A, ATP6V1B1). Most of the GTPase proteins identified belong to the small RAB G-protein protein sub-family which are involved in signal transduction and vesicle transport.[38] Notable examples identified in our proteome include RAB11A, RAB2A, RAB5A, GDI2, and CDC42.

Analysis of the biological processes enriched in ram seminal EVs revealed significant ($P < 0.05$) over-representation of GO terms associated with metabolism. Proteins involved in cell communication and adhesion were also highly represented in the proteome.

3.5. DAVID

DAVID functional annotation clustering was employed to give a high-level perspective of functional term enrichments. Six highly enriched clusters were returned which corresponded to three functional groupings (File 2, Supporting Information). The highest ranked cluster, which had an enrichment score of 7.0 [(-log (geometric mean of the enriched term *P*-values)], was represented by 12 terms relating to characteristics of a disintegrin and metalloprotease (ADAM) proteins. These included the terms metallopeptidase, disintegrin, EGF-like, and disulfide bond.

Annotation clusters 2, 3, and 4 with enrichment scores of 5.1, 3.3, and 2.6 respectively, were metabolism-related, and contained particular reference to proteins involved in glycolysis. GO terms associated with metabolism included carbon metabolism, gluconeogenesis, phosphoprotein, and oxidoreductase.

The third major class of proteins identified in ram seminal vesicles was associated with vesicular biogenesis and secretion. Cluster 6 (enrichment scores of 1.5) mapped to terms associated with vesicular biogenesis such as synaptic vesicle cycle, phagosome, and collecting duct acid secretion while Cluster 8 (enrichment scores of 1.3) mapped to small G-proteins of the Rab family containing terms linking to GTPase mediated signal transduction.

3.6. Vesiclepedia

The EV proteins identified in ram seminal plasma were compared with proteins previously published in exosomal studies and curated in the EV database, Vesiclepedia to gain insight into the commonality of the seminal EV proteome with EVs isolated from alternate extracellular fluids. The vesiclepedia database (www.microvesicles.org) contains over 92 000 protein entries from 538 studies and the human database (which was used as it contains the largest number of proteins for comparison) contained 8363 unique vesicular proteins at the time of analysis. Of the 116 proteins up-regulated in ram seminal EVs 89 were previously identified in this database indicating that there is substantial overlap with other EV studies. Unsurprisingly, the majority of the non-identified proteins ($n = 26$; indicated by an * in Table 1 and File 2, Supporting Information) were reproduction specific proteins and the majority of these originated in the testis (e.g., SLC9B1, PRSS21, PRSS42, CMTM2, ADAMs). This protein subset was also heavily enriched in GPI anchors with 42% of the 26 proteins returning a prediction for a GPI anchor (e.g., TEX101, DPEP3, and lymphocyte antigen 6 complex, LY6K) compared to only 15% in the total proteome. Many of the proteins not previously reported in Vesiclepedia have been shown to be essential for reproductive success in mice as they support sperm transit through the oviduct and sperm-zona binding (e.g., ADAM1, ADAM2, ADAM5, IZUMO1[39]).

3.7. Proteomic Characterization of the Ram Sperm Membrane Proteome

To provide further comparative information on the proteins shared between seminal plasma, extracellular vesicles, and the sperm membrane we comprehensively assessed proteins present in the ram sperm plasma membrane. Our strategy of combining sub-cellular fractionation techniques and 2D LC-MS/MS resulted in the identification of 462 proteins (≥2 peptides, FDR <1%; File 4, Supporting Information). Commonality between proteins in the extracellular vesicle population and the ram sperm membrane is indicated in Table 1 by an * in the "Sperm protein" column.

4. Discussion

In this study, SWATH was used as a quantitative proteomic tool to identify proteins that were enriched in extracellular vesicles isolated from ram seminal plasma. The quantitative comparison of two isolated extracellular vesicle bands with whole seminal plasma gave great insight into what proteins may be true vesicular proteins (e.g., Rabs, ADAMs) and what proteins may be co-isolated with extracellular proteins due to their adhesive nature (e.g., non-specific binding) or in vitro isolation (e.g., high speed centrifugation may pellet other cellular debris which associates with the EV population). For example, the highly abundant serum seminal plasma protein BSP5 was the ninth most abundant protein in the vesicular protein preparation but it was found at a concentration 20-fold less than whole seminal plasma, indicating it is likely to be a contaminant that is found in the EV population due to its high abundance in seminal plasma.

Gene ontology and network analysis of EV proteins showed ram seminal vesicles were enriched in proteins involved in vesicular biogenesis, metabolism and membrane adhesion, and remodeling. These functional groupings are shared across extracellular vesicles secreted from diverse cells and epithelium across a wide variety of species[40] but analysis of the individual proteins which underlie these functional groupings, reveals key differences which reflect species, tissue, and cell specificity/function. In our proteome, this was particularly true for the membrane adhesion and remodeling functional grouping that included many reproduction-specific proteins.

4.1. Ram Seminal EVs Contain a Reproduction-Specific Population of Extracellular Adhesion and Remodeling Proteins that are Closely Associated with Sperm Fertilizing Ability

Comparison of the ram seminal plasma EV proteome with previously published proteomes curated in the EV database, Vesiclepedia, highlighted a unique subset of reproduction-specific proteins that were previously not reported in the database ($n = 26/116$; indicated by an * in Table 1 and File 2, Supporting Information).

These proteins were extracellular proteins involved in sperm-specific surface remodeling and included ADAM proteins and proteins with a GPI-anchor that have been shown to be essential for fertility in mouse knockout models. The reproductive-specific ADAM proteins (a disintegrin and metalloproteinase domain-containing proteins) were also the most enriched functional cluster in ram seminal vesicles following DAVID analysis (File 2, Supporting Information). ADAM proteins have the potential to participate in cell adhesion and protease activities as they contain a disintegrin domain and a metalloprotease domain. Targeted remodeling of the extracellular matrix may occur through the release of cell adhesion proteins or signaling molecules (e.g., growth factors, cytokines) making ADAM proteins prime candidates for sperm surface remodeling in the male and female tract. The majority of ADAM proteins identified were testis localized proteins[41] that were also found on the ram sperm membrane (Table 1). The ADAM proteins identified in ram seminal vesicles (and ram spermatozoa), such as ADAM2 and proteins similar to ADAM5, ADAM20, and ADAM1A are not well described exosomal proteins and were not previously reported in the Vesiclepedia database (Table 1). Similar ADAM20-like proteins have been identified in macaque spermatozoa[42] but the gene has been lost in the mouse.[41] Testis expressed ADAM proteins are known to undergo proteolytic processing during epididymal maturation[43] and extracellular vesicles may aid this process. Two ADAMs (ADAM7 and ADAM28) of epididymal origin were also identified and these showed divergent localizations in seminal plasma. ADAM7 was 38-fold higher in the extracellular vesicle population whereas ADAM28 was 13-fold higher in seminal plasma (File 2, Supporting Information). As ADAM proteins contain a transmembrane domain their association with the sperm membrane or vesicular fraction of seminal plasma would be predicted. The exception to this is a splice variant of the ADAM28 gene, which lacks a transmembrane domain and is found as a soluble secretory protein.[44–46] The localization of ADAM28 in seminal plasma suggests this gene variant is present in ram seminal plasma.

The most abundant ADAM protein identified in our ram seminal EV proteome, ADAM7, has previously been shown to be expressed in the mouse epididymis[46,47] and transferred from the intracellular compartment of epithelial cells to the sperm surface by vesicular transport.[6] ADAM7 has also been shown to be associated with bull epididysomes and was expressed throughout the epididymis with the greatest expression in the corpus.[8] ADAM7 has not however, been identified in proteome studies of ram[49] (Table 1) or bull[35] spermatozoa indicating this protein may not be transferred to the sperm membrane in the epididymis in these species or it is removed prior to ejaculation. In comparison, a fibronectin protein ELSBP1, which is transferred from epididymosomes to bull spermatozoa, was highly enriched in the ram seminal EV protein preparation (36-fold higher concentration then whole seminal plasma) and identified in proteomic studies of epididymal and ejaculated spermatozoa[49] (Table 1) indicating the ram may share a similar vesicular mechanism of transport of this protein.

The other protein grouping in the ram seminal plasma EV proteome that had not been identified in previously published proteomes in the EV database, Vesiclepedia, contained proteins that had undergone post-translational modification to attach a GPI anchor (42% of the 26 proteins contained a putative GPI anchor compared to only 15% in the total proteome). Lipid-anchored proteins are located on the extracellular surface of the cell membrane and are preferentially located in subdomains of the plasma membrane, termed lipid rafts, which are involved in protein and lipid signaling and transport. Extracellular vesicles and lipid rafts both share the same distinctive lipid profiles that are characterized by an enrichment of cholesterol and sphingolipids.[49,50]

The GPI-anchored proteins identified in the ram seminal EV proteome have previously been shown to be essential for mouse fertility fertilization using KO mice experiments.[51] In particular the testis-expressed gene 101 (TEX101) and LY6K have been extensively studied. These proteins are members of the LU superfamily which is characterized by the presence of conserved cysteine residues and a putative GPI anchoring site.[52] In knockout mice the disruption of TEX101 leads to the removal of ADAM3 in the epididymis producing normal looking, but fertilization incompetent, spermatozoa.[53] The presence of TEX101 if thought to protect ADAM3 from proteolytic degradation in the epididymis. However, during epididymal transit TEX101 is itself shed from the sperm membrane and the impairment of this process results in the aberrant localization of ADAM3 on the sperm membrane which inhibits sperm migration into the oviduct and sperm-zona pellucida binding. Thus, both the presence and absence of TEX101 on the mouse sperm membrane, in the correct temporal-spatial manner, is essential for mouse sperm fertility. The angiotensin I converting enzyme (ACE) has been implicated in the shedding of TEX101 from the sperm membrane[53] and this protein is also enriched in our seminal plasma EV proteome and shown to be shed from the sperm membrane during epididymal transit in a range of domestic species which includes the ram.[54,55] Interestingly, while it has been reported that ACE has GPI-ase activity in spermatozoa[56] the mechanism by which ACE regulates the removal of TEX101 from the mouse sperm membrane appears to be independent of GPI-ase activity.[53] Indeed, the suggestion that ACE itself is directly responsible for the in vivo shedding of GPI-anchored proteins from the cell membrane has been described as doubtful with the alternate suggestion that ACE may be involved in the trafficking of GPI-anchored proteins or factors involved in their shedding.[57] Numerous other TEX101 interacting proteins were also enriched in the seminal EV proteome. The closely related protein, LY6K[58] has also been shown to be shed from the sperm membrane during epididymal transit and LY6K KO mice share the same infertile phenotype as TEX101 knockout mice.[59] Other notable example found in the seminal EV proteome include the SNARE protein VAMP3 which is thought to traffic TEX101 to the sperm surface during spermatogenesis[60] and the dipeptidase DPEP3 which has been shown to co-localize with TEX101 on testicular and epididymal spermatozoa but the function of this interaction is unclear.[61]

If these proteins showed similar expression and modification patterns in ovine models the enrichment of these group of interacting proteins in the vesicular fraction of SP could suggest the existence of a new role of extracellular vesicles in the male reproductive tract. Namely that extracellular vesicles not only deliver cargo to spermatozoa but may also aid in the removal or modification of sperm-bound proteins. As extracellular vesicles contain all the necessary cargo and biological mechanisms to target spermatozoa, dock to the sperm membrane and transfer proteins

from the vesicular membrane to the sperm membrane it is feasible that this process could occur in reverse. While there is much focus on the addition of proteins to the sperm membrane during epididymal maturation is it important to remember that the removal or loss of proteins from spermatozoa is equally important in the acquisition of fertilizing ability. In fact a recent proteomic investigation on the changes to the mouse spermatozoa during epididymal maturation showed that more proteins are potentially lost from spermatozoa ($n = 1034$) during transit, than added ($n = 732$).[62] The aforementioned infertile phenotype resulting from the inhibition of TEX101 removal from the sperm membrane by the KO of ACE is a clear example of the importance of protein shedding for sperm fertility.[53]

Last, there were two other membrane proteins in the ram seminal vesicle proteome that are worthy of mention. These were EGF-like repeat and discoidin I-like domain-containing protein 3 (EDIL3; also known as DEL-1) and Milk Fat Globule-EGF Factor 8 (MFGE8; also known as SED1 or lactadherin). These proteins are structural and functional homologs and they were among the most abundant proteins in the ram vesicular proteome. EDIL3 was the most abundant protein in ram seminal extracellular vesicles and MFGE8 the sixth most abundant (File 2, Supporting Information). MFGE8 has been shown to facilitate intercellular interactions between sperm and egg[63] and maintain the integrity of the epididymal epithelium[64] whereas the reproductive function of EDIL3 is unknown. Both proteins have been previously reported in extracellular vesicle from a range of somatic (MFGE8: dendritic cells,[65] osseous cells;[66] EDIL3: breast,[67] bladder,[68] and pancreatic[69] cancer cells) and reproductive cells (MFGE8: ram epididymal vesicles,[15] human seminal plasma EVs;[21] EDIL3 human seminal plasma EVs[21]). Recently, we showed through comparative proteomics of ejaculated and epididymal ram spermatozoa that only three novel proteins are added to the ram sperm membrane at ejaculation, EDIL3 being one of these three.[48] The relevance of this targeted binding of EDIL3 to the sperm membrane at ejaculation remains to be determined. Seminal extracellular vesicles have been previously shown to inhibit the lymphoproliferative response through a range of means[70–72] and seminal plasma has been shown to inhibit the binding of neutrophils to ram spermatozoa.[73] In endothelial cells EDIL3 has been shown to limit leukocyte recruitment during inflammation.[74] Thus, reason for the targeted inclusion of this vesicular seminal plasma to the ram sperm membrane at ejaculation may be to modulate the female immune system and aid sperm survival in the ewe reproductive tract.

4.2. Ram Seminal Plasma EVs are Enriched in Proteins Involved in Vesicle Formation, Trafficking, and Fusion

Many endosome-associated proteins involved in vesicle formation, trafficking, and fusion were enriched in the ram seminal EV proteome and their abundance in the vesicle population showed some of the highest fold-change increases compared to whole ram seminal plasma. These including RAB GTPases (e.g., RAB11A, RAB2A, RAB5A, GDI2, and CDC42), SNARE proteins (VAMP3, STX2), synaptophysins (SYPL1), and the synaptogyrin, SYNGR4 which showed the highest fold-change difference between the vesicular population and whole seminal plasma (278-fold higher in vesicle population; File 2, Supporting Information). There are three main mechanisms by which vesicles are released into the extracellular environment[75] i) budding of the vesicles directly from the plasma membrane to produce apical blebs (as observed in the epididymis), cell death leading to apoptotic bodies (which is unlikely to occur with spermatozoa as they undergo a truncated apoptotic pathway[76] and the exocytic release of multivesicular bodies (MVBs) formed from the inward budding of endosomal membranes (termed exosomes). Endosomal sorting complexes required for transport (ESCRT proteins 1–4) machinery and associated accessory proteins (ALIX, VPS4) are typically required for exosome biogenesis and intracellular transport and can act as markers to differentiate exosomes from apical blebs. No ECRT proteins were identified in the ram EV proteome which indicates these vesicles are a product of apical blebbing or MVBs are formed by a ESCRT independent mechanism, as has been reported in other cell lines.[77] It is also probable that the SWATH method used in this study, which focuses on protein quantification, rather than identification, failed to identity ESCRT proteins (and other proteins), which were present in the EV preparation. Regardless, it is likely that seminal plasma contains a heterogeneous population of vesicles that originate from the various locations along the male tract (epididymis, multiple accessory sex glands), through a variety of mechanisms. For this reason the all-encompassing term, extracellular vesicle, has been used in the current study rather than nomenclature that dictates the origin of the vesicle (e.g., epididymosomes, prostasomes) or its method of biogenesis (exosomes, apical bleb). It is also important to note that the vesicle formation, trafficking and fusion machinery identified above as essential for vesicle production and release is also the same machinery that would enable protein exchange (the removal or addition of proteins) at the sperm surface (e.g., membrane docking and fusion proteins such as SNAREs).[14,78]

4.3. Ram Seminal Plasma EVs are Enriched in Glycolytic Enzymes

Metabolic enzymes feature heavily in the top 100 proteins most commonly identified in extracellular vesicles[79] and key enzymes involved in anaerobic glycolysis were also highly enriched in ram seminal extracellular vesicles. These included HK1, PKM, LDHA, and TPI1 which were nine to 20-fold more abundant in seminal extracellular vesicles compared to seminal plasma (File 2, Supporting Information). Interestingly, two forms of The commonality of glycolytic enzymes across extracellular vesicles from a wide variety of species and from varying points of origin (e.g., epididysomes versus prostasomes) indicates the use of glycolysis for ATP production is well conserved. Indeed, seminal extracellular vesicles from a range of species have been shown to be capable of ATP production in the presence of glucose or fructose. However, the level of ATP production is quite varied with the bull showing markedly higher ATP production from seminal EVs compared to the dog.[80] Since dogs have well developed prostate glands, and the bull and the ram do not, it is tempting to speculate that this difference is due to a differing contribution of EVs from the prostate ("prostasomes") across the species. Interestingly, the

net production of ATP formation was balanced across the species by a higher functioning ATPase system in the bull. Numerous ATPase transporters were enriched in the ram SEVs which used ATP to transport phospholipids (ATP11C), sodium/potassium (ATP1A4, ATP1B3), and calcium (ATP2B1, ATP2B4). These results show SEVs are fully functioning metabolic units but the role, if any, of their metabolites on sperm function is unclear. The glycolytic machinery of seminal vesicles is thought to be surface located and thus extracellularly produced metabolites could act as a secondary sperm messengers in a paracrine fashion.[10] Or the glycolytic machinery or metabolites from EVs may be onboarded to spermatozoa as a novel way to obtain additional metabolic support. EVs derived from cancer-associated fibroblasts can supply amino acids to nutrient deprived cancer cells but.[81] EV uptake by spermatozoa may also be an energy dependent process as the inhibition of glycolysis decreased the internalization of human SEVs into normal prostate epithelial cells and prostate cancer cells.[82]

5. Conclusion

The quantitative comparison of proteins in whole ram seminal plasma with purified seminal extra vesicles has provided further indication of which proteins are vesicular proteins and which proteins may be co-isolated with vesicular preparations due to their adhesive nature and in vitro isolation techniques. Interrogation of this protein profile shows ram seminal EVs are enriched in proteins involved in vesicle biogenesis, metabolism and membrane adhesion, and remodeling. The latter group contained many reproduction-specific proteins that have been linked to fertility through proteomic investigation and KO mice studies.[51] Many of these membrane-bound proteins show testicular expression but are shed from the sperm surface during epididymal maturation (e.g., TEX101, LY6K). Their association with seminal EVs suggests that EVs may not only deliver protein cargo to spermatozoa but may also assist in the removal and or uptake of sperm membrane proteins.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors would like to acknowledge the assistance of Dr. Ben Crossett, Dr. David Maltby, and Dr. Angela Connolly from the University of Sydney, Mass Spectrometry Core Facility (MSCF), for their time, advice, and dedication. This work was supported by funding from the NSW Stud Merino Breeders Association Trust and Australian Wool Innovation Limited. Collaboration between the authors was assisted by a University of Sydney—Utrecht University Partnership Collaboration Award.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

EDIL3, epididysomes, microvesicles, prostasomes, sperm surface

Received: September 6, 2019
Revised: April 11, 2020
Published online: June 8, 2020

[1] T. Leahy, B. Gadella, *Reproduction* **2011**, *142*, 759.
[2] W. Nickel, *Eur. J. Biochem.* **2003**, *270*, 2109.
[3] J. L. Gatti, X. Druart, P. Syntin, Y. Guerin, J. L. Dacheux, F. Dacheux, *Biol. Reprod.* **2000**, *62*, 950.
[4] C. Legare, B. Berube, F. Boue, L. Lefievre, C. R. Morales, *Mol. Reprod. Dev.* **1999**, *52*, 225.
[5] C. H. Yeung, S. Schröter, A. Wagenfeld, C. Kirchhoff, S. Kliesch, D. Poser, G. Weinbauer, E. Nieschlag, T. Cooper, *Mol. Reprod. Dev.* **1997**, *48*, 267.
[6] J. S. Oh, C. Han, C. Cho, *Mol. Cells* **2009**, *28*, 441.
[7] I. Brody, G. Ronquist, A. Gottfries, *Upsala J. Med. Sci.* **1983**, *88*, 63.
[8] V. Thimon, G. Frenette, F. Saez, M. Thabet, R. Sullivan, *Human Reprod.* **2008**, *23*, 1698.
[9] K. M. Baska, G. Manandhar, D. Feng, Y. Agca, M. W. Tengowski, M. Sutovsky, Y. J. Yi, P. Sutovsky, *J. Cell. Physiol.* **2008**, *215*, 684.
[10] K. G. Ronquist, B. Ek, A. Stavreus-Evers, A. Larsson, G. Ronquist, *Am. J. Physiol., Endocrinol. Metab.* **2013**, *304*, E576.
[11] G. Frenette, J. Girouard, O. D'Amours, N. Allard, L. Tessier, R. Sullivan, *Biol. Reprod.* **2010**, *83*, 473.
[12] J. Girouard, G. Frenette, R. Sullivan, *Biol. Reprod.* **2009**, *80*, 965.
[13] G. Frenette, J. Girouard, R. Sullivan, *Biol. Reprod.* **2006**, *75*, 885.
[14] B. Nixon, G. N. De Iuliis, H. M. Hart, W. Zhou, A. Mathe, I. R. Bernstein, A. L. Anderson, S. J. Stanger, D. A. Skerrett-Byrne, M. F. B. Jamaluddin, J. G. Almazi, E. G. Bromfield, M. R. Larsen, M. D. Dun, *Mol. Cell. Proteomics* **2019**, *18*, S91.
[15] J.-L. Gatti, S. Métayer, M. Belghazi, F. Dacheux, J.-L. Dacheux, P. P. Identification, *Biol. Reprod.* **2005**, *72*, 1452.
[16] G. Ronquist, I. Brody, *Biochim. Biophys. Acta, Rev. Biomembr.* **1985**, *822*, 203.
[17] G. Ronquist, *J. Intern. Med.* **2012**, *271*, 400.
[18] A. G. Utleg, E. C. Yi, T. Xie, P. Shannon, J. T. White, D. R. Goodlett, L. Hood, B. Lin, *The Prostate* **2003**, *56*, 150.
[19] A. Poliakov, M. Spilman, T. Dokland, C. L. Amling, J. A. Mobley, *The Prostate* **2009**, *69*, 159.
[20] L. Dubois, B. Ek, G. Ronquist, A. Larsson, *Mol. Cell. Proteomics* **2015**, *14*, 3015.
[21] C. Yang, W.-b. Guo, W.-s. Zhang, J. Bian, J.-k. Yang, Q.-z. Zhou, M.-k. Chen, W. Peng, T. Qi, C.-y. Wang, *Andrology* **2017**, *5*, 1007.
[22] M. Aalberts, T. A. E. Stout, W. Stoorvogel, *Reproduction* **2014**, *147*, R1.
[23] R. Sullivan, F. Saez, *Reproduction* **2013**, *146*, R21.
[24] T. Leahy, S. P. de Graaf, *Reprod. Domest. Anim.* **2012**, *47*, 207.
[25] J. P. Rickard, T. Pini, C. Soleilhavoup, J. Cognié, R. Bathgate, G. W. Lynch, G. Evans, W. Maxwell, X. Druart, S. De Graaf, *Reproduction* **2014**, *148*, 469.
[26] T. Muino-Blanco, R. Perez-Pe, J. A. Cebrian-Perez, *Reprod. Domest. Anim.* **2008**, *43*, 18.
[27] J. M. Bedford, *Biol. Reprod.* **2015**, *92*, 1.
[28] R. El-Hajj Ghaoui, L. Gillan, P. C. Thomson, G. Evans, W. M. C. Maxwell, *J. Androl.* **2007**, *28*, 109.
[29] W. Zhou, G. N. De Iuliis, M. D. Dun, B. Nixon, *Front. Endocrinol.* **2018**, *9*, 59.
[30] R. J. Lobb, M. Becker, S. Wen Wen, C. S. Wong, A. P. Wiegmans, A. Leimgruber, A. Möller, *J. Extracell. Vesicles* **2015**, *4*, 27031.

[31] F. M. Flesch, W. F. Voorhout, B. Colenbrander, L. M. van Golde, B. M. Gadella, *Biol. Reprod.* **1998**, *59*, 1530.
[32] E. Sostaric, C. H. van de Lest, B. Colenbrander, B. M. Gadella, *Biol. Reprod.* **2005**, *72*, 346.
[33] G. Gillis, R. Peterson, L. Russell, L. Hook, M. Freund, *Prep Biochem* **1978**, *8*, 363.
[34] R. Peterson, L. Russell, D. Bundman, M. Freund, *Biol. Reprod.* **1980**, *23*, 637.
[35] K. Byrne, T. Leahy, R. McCulloch, M. L. Colgrave, M. K. Holland, *Proteomics* **2012**, *12*, 3559.
[36] J. Rickard, T. Leahy, C. Soleilhavoup, G. Tsikis, V. Labas, G. Harichaux, G. Lynch, X. Druart, S. de Graaf, *J. Proteomics* **2015**, *126*, 303.
[37] C. Soleilhavoup, G. Tsikis, V. Labas, G. Harichaux, P. Kohnke, J.-L. Dacheux, Y. Guérin, J.-L. Gatti, S. De Graaf, X. Druart, *J. Proteomics* **2014**, *109*, 245.
[38] A. H. Hutagalung, P. J. Novick, *Physiol. Rev.* **2011**, *91*, 119.
[39] N. Inoue, M. Ikawa, M. Okabe, *Asian J. Androl.* **2011**, *13*, 81.
[40] L. Rosa-Fernandes, V. B. Rocha, V. C. Carregari, A. Urbani, G. Palmisano, *Front. Chem.* **2017**, *5*, 102.
[41] C. Cho, *Nat. Rev. Urol.* **2012**, *9*, 550.
[42] S. Skerget, M. Rosenow, A. Polpitiya, K. Petritis, S. Dorus, T. L. Karr, *Mol. Cell. Proteomics 12*, 3052, **2013**.
[43] C. P. Blobel, D. G. Myles, P. Primakoff, J. M. White, *J. Cell Biol.* **1990**, *111*, 69.
[44] C. M. Roberts, P. H. Tani, L. C. Bridges, Z. Laszik, R. D. Bowditch, *J. Biol. Chem.* **1999**, *274*, 29251.
[45] L. Howard, R. A. Maciewicz, C. P. Blobel, *Biochem. J.* **2000**, *348*, 21.
[46] J. Oh, J.-M. Woo, E. Choi, T. Kim, B.-N. Cho, Z. Y. Park, Y. C. Kim, D. H. Kim, C. Cho, *Biochem. Biophys. Res. Commun.* **2005**, *331*, 1374.
[47] G. A. Cornwall, N. Hsia, *Endocrinology* **1997**, *138*, 4262.
[48] T. Pini, T. Leahy, C. Soleilhavoup, G. Tsikis, V. Labas, L. Combes-Soia, G. Harichaux, J. P. Rickard, X. Druart, S. P. de Graaf, *J. Proteome Res.* **2016**, *15*, 3700.
[49] J. F. Brouwers, M. Aalberts, J. W. A. Jansen, G. van Niel, M. H. Wauben, T. A. E. Stout, J. B. Helms, W. Stoorvogel, *Proteomics* **2013**, *13*, 1660.
[50] L. D. Zajchowski, S. M. Robbins, *Eur. J. Biochem.* **2002**, *269*, 737.
[51] M. Okabe, *Asian J. Androl. 17*, 646, **2015**.
[52] A. G. de Nooij-van Dalen, G. A. van Dongen, S. J. Smeets, E. J. Nieuwenhuis, M. Stigter-van Walsum, G. B. Snow, R. H. Brakenhoff, *Int. J. Cancer* **2003**, *103*, 768.
[53] Y. Fujihara, K. Tokuhiro, Y. Muro, G. Kondoh, Y. Araki, M. Ikawa, M. Okabe, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 8111.
[54] S. Métayer, F. Dacheux, J.-L. Dacheux, J.-L. Gatti, *Biol. Reprod.* **2002**, *67*, 1763.
[55] J. L. Gatti, X. Druart, Y. Guerin, F. Dacheux, J. L. Dacheux, *Biol. Reprod.* **1999**, *60*, 937.
[56] G. Kondoh, H. Tojo, Y. Nakatani, N. Komazawa, C. Murata, K. Yamagata, Y. Maeda, T. Kinoshita, M. Okabe, R. Taguchi, J. Takeda, *Nat. Med.* **2005**, *11*, 160.
[57] L. Leisle, E. T. Parkin, A. J. Turner, N. M. Hooper, *Nat. Med.* **2005**, *11*, 1139.
[58] H. Yoshitake, H. Tsukamoto, M. Maruyama-Fukushima, K. Takamori, H. Ogawa, Y. Araki, *Biochem. Biophys. Res. Commun.* **2008**, *372*, 277.
[59] Y. Fujihara, M. Okabe, M. Ikawa, *Biol. Reprod.* **2014**, *90*, 60.
[60] H. Tsukamoto, H. Yoshitake, M. Mori, M. Yanagida, K. Takamori, H. Ogawa, T. Takizawa, Y. Araki, *Biochem. Biophys. Res. Commun.* **2006**, *345*, 229.
[61] H. Yoshitake, M. Yanagida, M. Maruyama, K. Takamori, A. Hasegawa, Y. Araki, *J. Reprod. Immun.* **2011**, *90*, 202.
[62] S. Skerget, M. A. Rosenow, K. Petritis, T. L. Karr, *PLoS One* **2015**, *10*, e0140650.
[63] M. A. Ensslin, B. D. Shur, *Cell* **2003**, *114*, 405.
[64] A. S. Raymond, B. D. Shur, *J. Cell Sci.* **2009**, *122*, 849.
[65] P. Véron, E. Segura, G. Sugano, S. Amigorena, C. Théry, *Blood Cells Mol. Dis.* **2005**, *35*, 81.
[66] M. Balcerzak, A. Malinowska, C. Thouverey, A. Sekrecka, M. Dadlez, R. Buchet, S. Pikula, *Proteomics* **2008**, *8*, 192.
[67] J.-E. Lee, P.-G. Moon, Y.-E. Cho, Y.-B. Kim, I.-S. Kim, H. Park, M.-C. Baek, *J. Proteomics* **2016**, *131*, 17.
[68] C. R. Silvers, H. Miyamoto, E. M. Messing, G. J. Netto, Y.-F. Lee, *Oncotarget* **2017**, *8*, 91199.
[69] S.-H. Jiang, Y. Wang, J.-Y. Yang, J. Li, M.-X. Feng, Y.-H. Wang, X.-M. Yang, P. He, G.-A. Tian, X.-X. Zhang, Q. Li, X.-Y. Cao, Y.-M. Huo, M.-W. Yang, X.-L. Fu, J. Li, D.-J. Liu, M. Dai, S.-Y. Wen, J.-R. Gu, J. Hong, R. Hua, Z.-G. Zhang, Y.-W. Sun, *Oncotarget* **2015**, *7*, 4226.
[70] R. Tarazona, E. Delgado, M. C. Guarnizo, R. G. Roncero, S. Morgado, B. Sanchez-Correa, J. J. Gordillo, J. DeJulian, J. G. Casado, *Immunobiology* **2011**, *216*, 41.
[71] R. Kelly, P. Holland, G. Skibinski, C. Harrison, L. McMillan, T. Hargreave, K. James, *Clin. Exp. Immunol.* **1991**, *86*, 550.
[72] G. Skibinski, R. Kelly, D. Harkiss, K. James, *Am. J. Reprod. Immunol.* **1992**, *28*, 97.
[73] T. Pini, T. Leahy, S. P. de Graaf, *Reprod. Fert. Dev.* **2018**, *30*, 689.
[74] E. Y. Choi, E. Chavakis, M. A. Czabanka, H. F. Langer, L. Fraemohs, M. Economopoulou, R. K. Kundu, A. Orlandi, Y. Y. Zheng, D. A. Prieto, *Science* **2008**, *322*, 1101.
[75] S. Mathivanan, H. Ji, R. J. Simpson, *J. Proteomics* **2010**, *73*, 1907.
[76] R. J. Aitken, *Biol. Reprod.* **2011**, *85*, 9.
[77] K. Trajkovic, C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Brügger, M. Simons, *Science* **2008**, *319*, 1244.
[78] P. S. Tsai, N. Garcia-Gil, T. van Haeften, B. M. Gadella, *PLoS One* **2010**, *5*, e11204.
[79] H. Kalra, R. J. Simpson, H. Ji, E. Aikawa, P. Altevogt, P. Askenase, V. C. Bond, F. E. Borràs, X. Breakefield, V. Budnik, *PLoS Biol.* **2012**, *10*, e1001450.
[80] K. G. Ronquist, B. Ek, J. Morrell, A. Stavreus-Evers, B. S. Holst, P. Humblot, G. Ronquist, A. Larsson, *Biochim. Biophys. Acta, Gen. Subj.* **2013**, *1830*, 4604.
[81] H. Zhao, L. Yang, J. Baddour, A. Achreja, V. Bernard, T. Moss, J. C. Marini, T. Tudawe, E. G. Seviour, F. A. San Lucas, H. Alvarez, S. Gupta, S. N. Maiti, L. Cooper, D. Peehl, P. T. Ram, A. Maitra, D. Nagrath, *eLife* **2016**, *5*, e10250.
[82] K. G. Ronquist, C. Sanchez, L. Dubois, D. Chioureas, P. Fonseca, A. Larsson, A. Ullén, J. Yachnin, G. Ronquist, T. Panaretakis, *J. Extracell. Vesicles* **2016**, *5*, 29877.